Membrane Estrogen Receptor Regulates Experimental Autoimmune Encephalomyelitis through Up-regulation of Programmed Death 1

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Membrane Estrogen Receptor Regulates Experimental Autoimmune Encephalomyelitis through Up-regulation of Programmed Death 1

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Although estrogens exert a pronounced protective effect on multiple sclerosis and its animal model, experimental autoimmune encephalomyelitis (EAE), their therapeutic application has been limited by undesirable side effects thought to be mediated primarily through estradiol binding to intracellular estrogen receptor α. In this study, we found that signaling through the putative membrane estrogen receptor, G protein-coupled receptor 30 (GPR30), was sufficient to mediate protection against EAE, which was significantly impaired in GPR30 gene-deficient mice. Treatment with G-1, an agonist that selectively activates GPR30 without engagement of the intracellular estrogen receptors, retained the ability of estradiol to protect against clinical and histological EAE without estradiol-associated side effects, deviated cytokine profiles, and enhanced suppressive activity of CD4+ T regulatory cells through a GPR30- and programmed death 1-dependent mechanism. This study is the first to evaluate the protective effect of GPR30 activation on EAE, and provides a strong foundation for the clinical application of GPR30 agonists such as G-1 in multiple sclerosis. The Journal of Immunology, 2008, 182: 3294–3303.

Sex steroids and glucocorticoids have long been used in the clinic to control allergy, asthma, and autoimmune diseases due to their anti-inflammatory effects. Multiple sclerosis (MS) is a debilitating neurological autoimmune disease with higher incidence in women (1). However, MS relapse rates are decreased during late pregnancy (2, 3), and treatment with pregnancy levels of estradiol reduces CNS lesions (4, 5). We demonstrated previously (6, 7) that relatively low doses of 17β-estradiol (E2) and estradiol (E3) confer potent protection against clinical and histological signs of experimental autoimmune encephalomyelitis (EAE), an animal model of MS. However, clinical application of estrogens, particularly estradiol, in MS is limited by undesirable side effects ranging from the triggering of breast and uterine cancer to the loss of appetite, rapid weight gain, and fluid retention. Most estrogenic side effects are believed to be mediated by estradiol

Materials and Methods

Mice

C57BL/6 (B6) mice were purchased from the animal service at National Cancer Institute. The generation of GPR30-deficient (GPR30KO or Gper−/−), and the respective wild-type (WT) control mice from the colony was described before (14). PD-1 gene-deficient (PD−1KO) mice were obtained from T. Honjo (Kyoto University, Kyoto, Japan). GPR30KO mice were generated on the 129Sv background and backcrossed with B6 mice for six generations. PD−1KO strains were backcrossed with B6 mice for more than 10 generations. All mice used for experiment were age-matched (8- to 10-wk-old) female, and rested for at least 7 days before treatment or immunization. Animals were bred (for gene-deficient strains), housed, and cared for according to institutional guidelines in the animal resource facility at the Veterans Affairs Medical Center.
Reagents

Mouse (m) myelin oligodendrocyte glycoprotein (MOG)-35–55 peptide (MEGVWYRSPSRVHLYRNGK) was synthesized using solid-phase techniques and was purified by HPLC at Beckman Institute, Stanford University. Heat-killed Mycobacterium tuberculosis H37RA is a product of Difco. Pertussis toxin (PTX) was purchased from List Biological Laboratories. [3H]Thymidine was purchased from PerkinElmer. The 17β-estradiol (E2) and corn oil were purchased from Sigma-Aldrich. The 2.5 mg/60 day release E2 and vehicle (placebo) pellets were purchased from Innovative Research of America. G-1 powder was purchased from Cayman Chemical and packaged into slow-release pellets by Innovative Research of America. All fluorescein-labeled Abs were purchased from eBioscience. Trilogy Ag-unmasking reagent was purchased from Cell Signaling. Microbeads for MACS cell separation were purchased from Miltenyi Biotec. Luminex Bio-Plex mouse cytokine assay kit was purchased from Bio-Rad. Vector MOM Immunodetection peroxidase kit and Hematoxylin/Quinacrine (CQ) were purchased from Vector Laboratories. mAbs SMI32 and SMI312 were purchased from Covance. DakoCytomation liquid diaminobenzidine substrate is a product of DakoCytomation. Cytoxel XYL mounting medium is from Richard-Allan Scientific.

Induction of EAE

Mice were inoculated s.c. in the flanks with 0.2 ml of an emulsion containing 200 μg of mMOG-35–55 peptide and an equal volume of CFA containing 200 μg of heat-killed M. tuberculosis H37RA. On the same day and 2 days after the immunization, each mouse was infected i.v. with 75 and 200 ng of PTX, respectively. The mice were assessed daily for clinical signs of EAE 2–3 days after the immunization, each mouse was injected i.v. with 75 μg/ml mMOG-35–55 peptide for 48 h. Supernatants were then harvested and stored at −80°C until tested for cytokines. Culture supernatants were assessed for cytokine levels using a Luminex Bio-Plex mouse cytokine assay kit following manufacturer’s instructions. The following cytokines were determined in a single assay in three separate experiments: IL-1β, IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-6, IL-12, IL-13, and IL-17.

Pathology and immunohistochemistry

Intact spinal columns were removed from experimental and control groups of mice. The spinal cords were dissected after fixation in 4% paraformaldehyde, dehydrated, and embedded in paraffin before sectioning. To examine neuroinflammation, the sections were stained with H&E. To examine demyelination, the sections were stained with Luxol fast blue plus periodic acid Schiff (LFB-PAS). Immunohistochemistry was performed, as described (6, 17). Briefly, spinal cords were fixed in 4% paraformaldehyde (mass/volume in PBS (pH 7.4)) at 4°C for at least 48 h. The spinal cords were dissected out from the columns, cut into sections 1–2 mm in length from the sampled thoracic or lumbar cords, dehydrated, and embedded in paraffin blocks. Then, 10-μm-thick sections were cut from paraffin blocks and mounted onto precleared microscope slides. The sections were dewaxed and rehydrated sequentially by xylene (2 min), gradient ethanol (100, 95, and 85%; 2 min each), and PBS (5 min), and then cooked (120°C) in Ag-unmasking agent Trilogy for 10 min in a pressure steamer. The endogenous peroxidase activity was blocked with 3% hydrogen peroxide in tap water for 5 min. The sections were incubated 1 h in a working solution of mouse IgG blocking reagent from the VECTOM MOM Immunodetection peroxidase kit, and then incubated sequentially with the primary Ab (SMI312 1/3000 or SMI32 1/1000 diluted in MOM diluents) for 30 min, MOM biotinylated anti-mouse IgG reagent for 10 min, VECTASTAIN ABC reagent for 5 min, and DakoCytomation liquid diaminobenzidine substrate until sections turned light brown. The slides were counterstained with hematoxylin for 30–60 s to visualize nuclei, washed with tap water, dehydrated, and mounted with Cytoxel XYL mounting medium. The sections were analyzed by light microscopy after staining and recorded with a digital camera. The damaged areas were labeled out by hand and traced with Bioquant software (Bioquant). The numbers of injured axons were counted by Scion Image.

Statistical analysis

Mean values from each experiment were compared statistically. Differences in group daily clinical scores, peak scores, and cumulative disease index (CDI) were evaluated by Kruskal-Wallis test, followed by Mann-Whitney (unpaired Student’s t test was used for daily EAE score or CDI if none of the mice were sick in one of the comparison groups); incidence was evaluated by Fisher’s exact test; disease onset, uterine, and body and femur weights were measured by one-way ANOVA, followed by Newman-Keuls test; endogenous steroid levels in serum or cerebrospinal fluid (CSF) were assessed using radioimmunoassay, as described previously (6, 17). Briefly, 4 million cells were surface stained following standard procedures. After washing, the cells were fixed overnight and washed twice with 0.5 ml of permeabilization buffer. The cells were then incubated with 2 μl of permeabilization buffer and one with 1 ml of staining buffer, and resuspended in staining buffer. Flow cytometry data were collected on LSRII and FACSCalibur flow cytometers (BD Biosciences), and analyzed using FlowJo software (Tree Star). Data represent 50,000–100,000 events, unless otherwise noted.

Lymphocyte proliferation assay

Splenocytes or T cells were harvested and cultured in a 96-well flat-bottom tissue culture plate at 4 × 10⁴ cells/well in stimulation medium in the presence of APCs, irradiated (2500 rad) syngeneic thymocytes at a ratio of 1:10 (T:APCs), either with or without mMOG-35–55 peptide at varying concentrations. The cells were incubated for 3 days at 37°C in 5% CO2 and pulsed with 0.5 μCi of [3H]Thymidine for the final 18 h of incubation. The cells were harvested onto glass fiber filters, and incorporated radioactivity was measured by a liquid scintillation counter. The cpm values (mean ± SD) were calculated. The calculation of the triplicate cell proliferation index was calculated by dividing the experimental cpm by the control cpm.

Cytokine determination by Luminex kit

Lymph node (LN) and spleen cells were cultured at 4 × 10⁶ cells/well in a 24-well flat-bottom culture plate in stimulation medium (RPMI 1640, 1% sodium pyruvate, 1% l-glutamine, 0.4% 2-MA, and 10% FBS) with 25 μg/ml mMOG-35–55 peptide for 48 h. Supernatants were then harvested and stored at −80°C until tested for cytokines. Culture supernatants were assessed for cytokine levels using a Luminex Bio-Plex mouse cytokine assay kit following manufacturer’s instructions. The following cytokines were determined in a single assay in three separate experiments: IL-1β, IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-6, IL-12, IL-13, and IL-17.

Results

GPR30 is sufficient for E2-induced protection against EAE

The contribution of GPR30 to E2-induced protection against EAE was evaluated in age-matched WT C57BL/6, GPR30KO, and hetrozygous (GPR30+/−) female mice from the same colony. Mice were implanted with E2 (2.5 mg/60-day release) or placebo pellets 1 wk before immunization with mMOG-35–55 peptide in CFA with additional PTX given on days 0 and 2. Serum E2 levels were 1.5–2 ng/ml (Fig. 3). As shown in Fig. 1A and Table I, placebo-treated WT and GPR30KO mice developed severe EAE with onset on day 11 and peak about day 15, whereas E2-treated WT mice developed no signs of disease. In contrast, E2-treated GPR30KO and hetrozygous mice had delayed onset and were only partially protected from EAE. Pathologically, all of the placebo-treated mice had substantial immune cell infiltration and demyelination in the CNS (Fig. 1B), whereas no pathological signs of EAE were found in E2-treated WT mice. In E2-treated GPR30KO mice, however, cellular infiltration and demyelination were visibly present, but at a reduced level. T cell responses to MOG peptide were similarly reduced by E2 treatment in both WT and GPR30KO...
mice (Fig. 1C), suggesting that GPR30 is not directly linked to suppression of T cell proliferation. Taken together, these results indicate a significant, but not exclusive role for GPR30 in E2-mediated protection against EAE.

Activation of GPR30 confers protection against clinical EAE

Although the above results indicated an indispensable role for GPR30 in E2-mediated protection against EAE, we sought to determine whether activation of GPR30 alone could provide protection. We thus compared the efficacy of G-1, the only specific GPR30 agonist available, and E2 in protecting wild-type C57BL/6 mice against EAE. Initially, G-1 (1.8, 0.1, or 0.01 mg/40-day release pellets), E2 (2.5, 0.1, or 0.025 mg/60-day release pellets), or placebo was administered to mice 7 days before induction of EAE. Administration of either 1.8 mg/40-day release G-1 or the molar equivalent level of 2.5 mg/60-day release E2 pellets completely protected the mice from clinical EAE, with lower doses of G-1 being less effective than E2 (Fig. 2A and Table II). The s.c. injections of G-1 (20 μg/day in corn oil) and E2 (1 μg/day in corn oil) were less protective against EAE (Fig. 2B and Table II) when compared with pellets. G-1 and E2 treatment nominally lowered ex vivo T cell proliferation responses to mMOG-35–55 peptides in this particular experiment, but statistical significance was not reached (Fig. 2C). Therefore, we conclude that G-1,

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** E2-induced protection against EAE is reduced in GPR30KO mice. Age-matched GPR30KO, WT, and heterozygous mice from the same colony were implanted with 2.5 mg/60-day release E2 or placebo pellets 1 wk before immunization with mMOG-35–55 peptide plus CFA and PTX. The mice were scored for EAE development and euthanized 25 days after immunization. A, The protective effect of E2 was reduced, but not completely abrogated in GPR30KO mice. B, E2 did not prevent CNS infiltration and demyelination in GPR30KO mice. For quantification, five mice randomly selected from each group were included, and similar differences were noted consistently in all mice from each group. C, GPR30 was not directly involved in suppression of T cell proliferation to mMOG-35–55 peptides. The cpm of control/10057199.5 ± 1080.6 (Spleen) or 5617.1 ± 665.1 (LN). *, p < 0.05 or **, p < 0.01, as compared with placebo control. The experiment was repeated three times with at least seven mice in each group.

Table I. Gene disruption of GPR30 weakens the therapeutic effects of E2 on EAE

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Pellet</th>
<th>n</th>
<th>Incidence</th>
<th>Mortality</th>
<th>Onset</th>
<th>Peak</th>
<th>Daily</th>
<th>CDI</th>
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<td>WT</td>
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<td>0 of 0</td>
<td>15.3 ± 2.1</td>
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<td>0**</td>
<td>0**</td>
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<td>0**</td>
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<td>GPR30KO</td>
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<td>0 of 7</td>
<td>13.4 ± 0.53</td>
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<td>3.6 ± 0.9</td>
<td>40.1 ± 13.2</td>
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<td>0 of 7</td>
<td>18.0 ± 1.4**</td>
<td>2.5 ± 2.3*</td>
<td>3.7 ± 1.3</td>
<td>17.2 ± 17.4**</td>
</tr>
<tr>
<td>GPR30/−/−</td>
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<td>0 of 10</td>
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<td>3.6 ± 1.6</td>
<td>3.5 ± 0.6</td>
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</tr>
<tr>
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<td>0 of 10</td>
<td>20.0 ± 1.0**</td>
<td>2.1 ± 2.3**</td>
<td>2.6 ± 0.8</td>
<td>11.3 ± 13.0**</td>
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</tbody>
</table>

* Mice were immunized 1 wk after implantation with 2.5 mg/60-day release E2 pellets. The experiment was concluded 25 days after immunization.

*, p < 0.05 or **, p < 0.01 as compared with placebo control; *, p < 0.05 as compared with E2-treated WT.
FIGURE 2. Activation of GPR30 conferred substantial protection against clinical EAE in WT B6 mice. A, Treatment with G-1 delayed and ameliorated EAE in a dose-dependent manner. Various doses of G-1, E2, or placebo pellets were imbedded underneath the skin on the back of the mice. One week following the start of treatment, the mice were immunized with mMOG-35–55 peptide plus CFA and PTX. The mice were scored for EAE development and euthanized 34 days after immunization for ex vivo studies. Values of \( p < 0.05 \) or 0.01 for 2.5 and 0.1 mg of E2 and 3.7 mg of G-1 treatment groups from days 14 to 34, and for 0.1 mg of G-1 treatment group from days 14 to 19 and from days 25 to 34. The experiment was repeated twice with five to eight mice in each group. B, G-1 injected s.c. had nominally less protection than E2 against EAE. G-1 (20 mg/mouse/day in 100 l of 10% ethanol and 90% olive oil), E2 (1 mg/mouse/day in 100 l of 10% ethanol and 90% olive oil), or placebo (100 l of 10% ethanol and 90% olive oil) was injected daily underneath the neck skin of the mice 1 wk before immunization. The mice were monitored for changes in clinical EAE scores and euthanized 20 days after the immunization. Values of \( p < 0.05 \) or 0.01 for E2 treatment groups from days 11 to 20, and for G-1 treatment group from days 11 to 17, as indicated by one-way ANOVA, followed by Newman-Keuls multiple comparisons test. C, T cell proliferation to mMOG-35–55 from G-1-, E2-, and placebo-treated mice. Splenocytes and LN cells were obtained ex vivo from G-1- or E2-implanted mice, as depicted in A, and incubated with various concentrations of antigenic peptides. Statistical difference was not reached among different treatment groups. D, G-1 but not E2-induced protection against EAE was abrogated in GPR30KO mice. Neither G-1 nor E2 affected T cell proliferation to mMOG-35–55. GPR30KO mice were immunized 1 wk after implantation with 1.8 mg/40-day release G-1 or 2.5 mg/60-day release E2 pellets for a week. The experiment was concluded 29 days after immunization for ex vivo experiments. *, \( p < 0.05 \) or **, \( p < 0.01 \), compared with placebo control. The experiment was repeated twice with 7–10 mice in each group. E, G-1 treatment did not increase the uterine weight in B6 mice. At the end of the clinical experiment, the weights of the whole mice, uteri, and femurs were measured. *, \( p < 0.05 \) compared with placebo control.
tested (Fig. 3), thus ruling out the possibility that up-regulation of E2. The results showed that G-1 slightly lowered the level of corticosteroid in sera from mice treated with placebo, 3.7 mg of G-1, or 2.5 mg of E2 (data not shown). To rule out the possibility that G-1 prevented H&E staining in liver, eyes, heart, mammary gland, brain, spleen, kidney, muscle, or lung from 1.8 mg of G-1-treated naive mice (data not shown). To rule out the possibility that G-1 prevented EAE by regulating endogenous steroid hormones, levels of E2, progesterone, or testosterone, and only endogenous anti-inflammatory steroid hormones was responsible for the clinical improvement caused by G-1 treatment.

**G-1 treatment reduced CNS inflammation, demyelination, and axonal damage**

Consistent with the clinical observations, both G-1 and E2 treatments markedly ameliorated immune cell infiltration and demyelination in the CNS, as indicated by H&E and LFB-PAS staining (Fig. 4, upper panels). Moreover, both agents reduced the level of axonal damage in spinal cord sections (Fig. 4, lower panels). Existing axons can be visualized by immunohistochemical staining with SMI312, an Ab mixture that stains phosphorylated (healthy) neurofilaments (NFL), and the degree of ongoing damage can be seen by staining nonphosphorylated NFL with Ab SMI32, which specifically detects injured and demyelinated axons (19, 20). In placebo-treated WT mice, axonal staining was markedly reduced in the presence of inflammatory mononuclear cells, resulting in severe loss of SMI312 staining in the outer region of white matter, where most neuroinflammation occurred (Fig. 4, lower left panel). In contrast, axons in the spinal cords of G-1- and E2-treated mice were well preserved. Additionally, sections from both G-1- and E2-treated mice showed much less SMI32 staining in the white matter of the spinal cords (Fig. 4, lower right panel). Thus, G1 and E2 induced comparable neuroprotective effects in EAE.

When administered alone at relatively high doses, was sufficient to induce complete protection against clinical EAE without observable effects on proliferation of Ag-specific T cells. Although G-1 was reported previously to bind specifically to GPR30 (18) and not to any of the iERs in vitro, its specificity has not yet been characterized in vivo. Using GPR30KO mice, we tested critically whether GPR30 is required for G-1-induced protection against EAE. GPR30 mice were implanted with placebo, G-1, or E2 pellets 7 days before immunization. Although the protective effect of E2 against EAE was only partially offset by the absence of GPR30, treatment with G-1 was completely ineffective in GPR30KO mice (Fig. 3D and Table III). Therefore, G-1-induced protection against clinical EAE was a result of specific activation of GPR30.

Of importance, treatment with G-1 in vivo lacked the estrogenic effects of E2. In contrast to E2, G-1 treatment did not significantly change the weight of uteri, a prominent and well-known estrogenic effect (Fig. 2E). Neither G-1 nor E2 significantly changed the body and femur weights. Additionally, there were no abnormalities by H&E staining in liver, eyes, heart, mammary gland, brain, spleen, kidney, muscle, or lung from 1.8 mg of G-1-treated naive mice (data not shown). To rule out the possibility that G-1 prevented EAE by regulating endogenous steroid hormones, levels of E2, progesterone, testosterone, and corticosteroid were measured in sera from mice treated with placebo, 3.7 mg of G-1, or 2.5 mg of E2. The results showed that G-1 slightly lowered the level of corticosteroid, but did not affect any of the other steroid hormones tested (Fig. 3), thus ruling out the possibility that up-regulation of endogenous anti-inflammatory steroid hormones was responsible for the clinical improvement caused by G-1 treatment.

**FIGURE 3.** G-1 treatment did not change the serum levels of E2, progesterone, or testosterone, and only slightly lowered corticosteroid. Steroid levels were measured in sera collected from placebo-, 3.7 mg of G-1-, and 2.5 mg of E2-treated mice used in clinical experiments shown in Fig. 2A. *, *p < 0.05 or ***, p < 0.001.

### Table II. The protective effects of G-1 vs E2 in WT B6 mice

<table>
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<tr>
<th>Treatment</th>
<th>n</th>
<th>Incidence</th>
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<td>13.7±1.1</td>
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<td>10.5±13.9**</td>
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* Mice were immunized 1 wk after implantation with G-1 or E2 pellets at different doses. The experiment was concluded 34 days after immunization. *p < 0.05 or ***, p < 0.01 as compared with placebo controls.
Our previous results showed that E2 treatment increased the percentage of CD4^+FoxP3^+ Treg cells and PD-1 expression within this cell population (6, 16, 21). We thus studied whether GPR30 is required for E2-induced up-regulation of Foxp3 or PD-1 expression in Treg cells. Splenocytes were harvested from placebo- or E2-treated WT and GPR30KO mice (from Fig. 1A) and stained for CD4, FoxP3, and PD-1. The results showed that GPR30 was not required for E2-induced up-regulation of CD4^+FoxP3^+ T cells (data not shown). However, E2-induced up-regulation of PD-1 expression in these cells was abolished in splenocytes from GPR30KO mice (Fig. 5A). E2 did not induce a significant shift in

**FIGURE 4.** G-1 treatment reduced CNS infiltration (H&E), demyelination (LFB-PAS), axonal loss (NFL), and ongoing axonal damage (dephosphorylated NFL). The mice from the clinical experiment shown in Fig. 2A were euthanized at the end of the experiment, and spinal cords from ≥3 mice from each group were dissected for histology. Immune cell infiltration and demyelination of CNS were examined with H&E and LFB-PAS staining. Total and damaged axons were examined with immunohistological staining for NFL or dephosphorylated NFL.

GPR30 up-regulates PD-1 in T regulatory (Treg) cells

Our previous results showed that E2 treatment increased the percentage of CD4^+FoxP3^+ Treg cells and PD-1 expression within this cell population (6, 16, 21). We thus studied whether GPR30 is required for E2-induced up-regulation of Foxp3 or PD-1 expression in Treg cells. Splenocytes were harvested from placebo- or
PD-1 expression in the nonregulatory CD4+FoxP3+ T cells in WT or GPR30KO mice. Therefore, we conclude that GPR30 is required for E2-induced up-regulation of PD-1 in Treg cells.

We then tested whether activation of GPR30 up-regulates PD-1 in CD4+FoxP3+ Treg cells. Splenocytes were harvested from placebo-, E2-, or G-1-treated mice after EAE induction and stained for CD4, FoxP3, and PD-1. Approximately half of the CD4+ FoxP3+ T cells from the spleen of placebo-treated mice were negative or low for PD-1 expression (Fig. 5B). Of critical importance, both E2 and G-1 treatments strongly enhanced the staining intensity of PD-1 and converted a majority of the CD4+FoxP3+PD-1− cells to CD4+FoxP3+PD-1+ cells. Surprisingly, G-1 was even more potent than E2 in boosting PD-1 expression in CD4+FoxP3+ cells, suggesting that the iER pathways for E2 might have opposing effects on GPR30. Functional assays using FoxP3-GFP knock-in mice indicated that GFP+PD-1− Treg cells were more suppressive than GFP+PD-1− Treg cells (C. Wang, Y. Li, T. M. Proctor, A. A. Vandenbark, and H. Offner, submitted for publication). Thus, activation of GPR30 may cause Treg cells to shift from PD-1− to PD-1+ with enhanced suppressive function.
PD-1 is critical to G-1-induced EAE protection and decrease of IL-17 production

To evaluate whether G-1-induced up-regulation of PD-1 is important for EAE protection, PD-1KO mice were implanted with 2.5 mg/60-day release E2, 1.8 mg/40-day release G-1, or placebo pellets 1 wk before immunization to induce EAE. As shown in Fig. 6A and Table IV, G-1 failed to protect against clinical EAE in PD-1KO mice, whereas E2 retained partial efficacy. In addition, G-1 treatment did not significantly change T cell proliferation (B) and cytokine secretion in 48-h supernatants (C). *, p < 0.05 or **, p < 0.01 compared with placebo. The experiment was repeated twice with a total of 7–10 mice in each group.

Lastly, we evaluated the effects of G-1 treatment and PD-1 expression on cytokine profiles in splenocytes harvested from G-1- or placebo-treated WT and PD-1KO-immunized mice. As shown in Fig. 6C, G-1 treatment significantly reduced secretion of IL-17 and IL-2, critical proinflammatory cytokines that play a vital role in EAE induction (22), and increased the secretion of IL-10, a key anti-inflammatory cytokine. Interestingly, G-1 also increased the production of IFN-γ, a hallmark Th1 cytokine, as well as IL-6, and suppressed IL-4, but did not significantly change the production of any other cytokines examined, including IL-1β, IL-5, IL-12, and TNF-α. Of importance, this cytokine profile was drastically altered...

Table III. The protective effect of G-1 was abrogated in GPR30KO micea

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Incidence</th>
<th>Mortality</th>
<th>Onset</th>
<th>Peak</th>
<th>Daily</th>
<th>CDI</th>
</tr>
</thead>
<tbody>
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<td>14.7 ± 1.5</td>
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<td>2.5 ± 1.1</td>
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<td>0 of 8</td>
<td>13.8 ± 1.2</td>
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<td>3.0 ± 0.7</td>
<td>59.9 ± 13.4</td>
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<td>E2</td>
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<td>4 of 7</td>
<td>0 of 7</td>
<td>18.0 ± 1.4**</td>
<td>2.5 ± 2.3*</td>
<td>3.7 ± 1.3</td>
<td>17.2 ± 17.4**</td>
</tr>
</tbody>
</table>

a GPR30KO mice were immunized 1 wk after implantation with 1.8 mg/40-day release G-1 or 2.5 mg/60-day release E2 pellets for 1 wk. The experiment was concluded 29 days after immunization. *, p < 0.05 or **, p < 0.01 as compared with placebo control. *, p < 0.05 as compared with G-1-treated mice.
in G1-treated PD-1KO mice. The decrease of IL-17 and increase of IL-6, IL-10, and IFN-γ production were abolished, whereas the reduction in IL-2 and IL-4 remained. Thus, PD-1 was required for GPR30 down-regulation of IL-17, but not IL-2 and IL-4, and up-regulation of IL-6, IL-10, and IFN-γ. The result that G1 treatment reduced IL-17 production through a PD-1-dependent mechanism is of particular importance because this cytokine has been closely linked to neuroinflammation in EAE.

Discussion

The results presented above demonstrate for the first time that the mER, GPR30, is both necessary and sufficient for full E2-mediated protection against EAE. Moreover, the agonist G1, which selectively activates GPR30 without engagement of the iERs, retained the ability of E2 to protect against clinical and histological EAE without obvious estrogenic side effects, including increased uterine weight. The prospect of using estrogens to treat autoimmune diseases such as MS has been complicated by considerable side effects and risk, which are believed to be associated with transcription-modifying functions of iERs. Our study thus demonstrates possible clinical significance of G1 activation of GPR30 and the selective up-regulation of PD-1 by CD4+Foxp3+ Treg cells.

In this study, we used the GPR30KO mouse model to define whether GPR30 is indispensable for E2-mediated full protection against EAE. Our result indicated that GPR30 is an active contributor, but not the only player in mediating the suppressive effects of E2 on EAE. This conclusion is consistent with our previous results on iER gene-deficient mice (23), which showed that the protective effect of E2 was mainly dependent on ERα. In that study, although E2 treatment delayed the disease onset for 4 days, it did not reduce the incidence, disease peak, or CDI score. Similarly, Morales et al. (31) reported complete loss of protection by estriol treatment in ERKO mice. However, as shown in Fig. 1, there clearly is residual E2 protection in GPR30KO mice, indicating contribution of other receptors, presumably ERαs. Thus, it is likely that both GPR30 and ERα participate in E2-mediated protection in an additive manner. However, based on the difference in residual protection in ERKO vs GPR30KO mice treated with E2, we think that there is a lesser contribution of GPR30 to protection when compared with ERαs. We cannot yet conclude that E2-induced protection against EAE is mediated exclusively by ERα plus GPR30, but GPR30KO and ERKO double-gene-deficient mice are currently being constructed to address this issue.

Because our result in GPR30KO mice indicated that GPR30 contributed to E2-mediated protection against EAE, we determined whether activation of GPR30 alone could confer protection against EAE. The development of selective agonists for mER made it possible to specifically activate mER without trans activation of iERs. Qiu et al. (24) described a synthesized compound, STX, that does not exhibit any binding affinity for the nuclear receptors ERα or ERβ, but mimics the quick effect of estrogens on neurons. Yet, the molecular target of STX has not been identified, and it remains to be determined whether STX indeed interacts with a mER. Besides STX, the only other reported mER ligand is G1, which we selected for further study due to its ability to bind GPR30 with high affinity and excellent specificity (25). Remarkably, none of the mice receiving the highest dose G1 pellets showed any clinical signs of EAE. In the absence of GPR30, the effect of G1, but not E2, completely disappeared. These results suggested that solo activation of GPR30 is sufficient to confer protection against EAE. The protective effects of G1 in WT mice eliminated the possibility that the observed role of GPR30 could be due merely to an artifact of gene deletion or a compensation effect in the developing animal.

How could G1 treatment at a sufficiently high dose completely protect mice against EAE, whereas GPR30 is only one of the receptors that mediate the protective effect of E2 against EAE? Because G1 did not protect the mice from EAE in GPR30KO mice, we do not believe that trans activation of ERαs by G1 played a significant role. Also, we did not detect any significant increase in the serum levels of estradiol, corticosterone, progesterone, or testosterone in G1-treated mice, thus ruling out the possibility that up-regulation of endogenous steroid hormones accounted for the clinical and neuroprotective effects of G1 treatment. Thus, we conclude that ERα and GPR30 work together additively to achieve optimal protection, and each receptor may adequately compensate the functional loss of the other.

Mechanistically, we observed that G1 treatment did not directly suppress pathogenic T cell proliferation, nor did it increase the percentage of CD4+Foxp3+ Treg cells. Nevertheless, both E2 and G1 markedly increased the level of PD-1 expression in CD4+Foxp3+ Treg cells. PD-1 has been found to play a crucial role in the development and maintenance of peripheral tolerance (26). In a separate study, we sorted PD-1+ and PD-1− Treg cells from Foxp3-GFP knock-in mice and found that PD-1− Treg cells had increased suppressive function (C. Wang, Y. Li, T. M. Proctor, A. A. Vandenbark, and H. Offner, submitted for publication). Moreover, the therapeutic effect of G1, but not E2, disappeared completely in PD-1KO mice. Thus, it seems that G1 is exclusively dependent on the presence of PD-1 to function, but the effects of E2 could be mediated by alternative pathways in the absence of PD-1. Taken together, these results suggest that G1 may suppress EAE by up-regulation of the PD-1 signaling pathway in CD4+Foxp3− cells.

The result that both E2 and G1 decrease IL-17 in vivo is remarkable because this cytokine has been closely linked to the development of EAE. That G1 failed to reduce IL-17 production in PD-1 gene-deficient mice strongly supports the idea that G1 regulates IL-17 production via manipulation of PD-1. Increased secretion of the prototypic Th1 cytokine, IFN-γ, seemed to be in conflict with the fact that G1 ameliorated disease severity and tissue damage. Unlike IL-17, however, IFN-γ appears to be pleiotrophic. Although administration of IFN-γ worsened symptoms of MS (27), it improved EAE in mice (28–30), and IFN-γ gene-disrupted mice were more susceptible to the induction of EAE (29). Thus, increased production of IFN-γ may actually facilitate the containment of EAE in some circumstances. However, our previous results indicated that E2 treatment reduced IFN-γ (23), raising the possibility that effects of E2 and G1 may differ.

Table IV. The protective effect of G1, but not E2, was abolished in PD-1KO mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Incidence</th>
<th>Mortality</th>
<th>Onset</th>
<th>Peak</th>
<th>Daily</th>
<th>CDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
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<td>5 of 5</td>
<td>1 of 5</td>
<td>12.2 ± 0.8</td>
<td>5.1 ± 0.2</td>
<td>3.5 ± 0.5</td>
<td>38.9 ± 5.1</td>
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<tr>
<td>G1</td>
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<td>5 of 5</td>
<td>1 of 5</td>
<td>13.8 ± 1.3</td>
<td>4.8 ± 0.6</td>
<td>2.7 ± 0.8</td>
<td>30.6 ± 9.1</td>
</tr>
<tr>
<td>E2</td>
<td>5</td>
<td>5 of 5</td>
<td>0 of 5</td>
<td>16.4 ± 0.9*</td>
<td>4.1 ± 0.8</td>
<td>1.5 ± 0.6*</td>
<td>16.4 ± 6.3**</td>
</tr>
</tbody>
</table>

* PD-1KO mice were immunized 1 wk after implantation with 1.8 mg/40-day release G1 or 2.5 mg/60-day release E2 pellets for 1 wk. ** p < 0.05 or *** p < 0.01 as compared with placebo control.
with regard to IFN-γ production and its effect on protection against EAE.

Our results showed that treatment with G-1 in vivo lacked some of the prominent estrogenic effects of E2 and did not cause any abnormalities in liver, eyes, heart, mammary gland, brain, spleen, kidney, muscle, or lung. Although we believe that the use of GPR30 ligands will avoid some of the side effects mediated by iERα, it remains to be seen whether or not GPR30 agonists represent a safer alternative to estrogen treatment. For instance, GPR30 may play an important role in promoting breast and uterine cancer progression (12). Thus, G-1 signaling through GPR30 might be as potent as estradiol signaling through iERα with respect to carcinogenesis. Nevertheless, our study demonstrated that it is possible to avoid some of the side effects mediated through iERs by specifically targeting the membrane receptor, although retaining much of the therapeutic efficacy of estradiol, at least in EAE.

Taken together, we showed that the putative mER, GPR30, is sufficient, yet not exclusively responsible for full E2-mediated protection against EAE. Treatment with G-1 that specifically targets GPR30 suppressed clinical and histological EAE and the production of IL-17 by up-regulation of PD-1 expression in regulatory T cells. This study is the first to evaluate the contribution of a membrane steroid receptor in suppression of autoimmune disease in an animal model, and may provide the necessary foundation for the clinical application of membrane steroid receptor agonists such as G-1 in human subjects.

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Disclosures

The authors have no financial conflict of interest.

References